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The Mode of Occurrence of Carotene and Vitamin A in Human Blood Plasma

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This work was undertaken primarily in the hope that a knowledge of the way in which carotene and vitamin A are carried in blood would aid in solving the problem of the apparently erratic occurrence of vitamin A in abnormal urines.

The existing statements are conflicting, and the evidence in support of them is scanty. Palmer (1915), impressed by the impossibility of extracting more than traces of carotene from cow serum by ethanol-free ether, and by the different behaviour of cow and hen sera, postulated a carotene-albumin compound. Pett & Le Page (1940) believed that their experiments on fractional precipitation showed the carotene to be bound to serum globulin, and Bennhold's (1938) measurements of cataphoretic migration support this view. On the other hand, Van den Bergh & Müller (1920) showed that simple aqueous colloidal solutions of carotene resembled serum in that the carotene was not readily extracted by organic solvents such as ether and, though there were some differences, they considered carotene as existing in serum in simple colloidal solution. Their observations were confirmed by Drummond & McWalter (1935). The existence in nature of carotene- or carotenoid-proteins has been demonstrated, e.g. lycopene-protein in tomato (Kuhn & Bielg (1940), astacene-protein and rhodopsin, vitamin A-protein (Wald, 1938; Lovern & Morton, 1939), ovoidin, astaxanthin-protein (Kuhn & Sorensen,

1938). However, the fact that these substances exist, though it shows the possibility, does not indicate whether or not carotene and vitamin A are actually carried in blood plasma as protein (or other) compounds or in simple colloidal solution.

METHODS

The plan was to examine the power of ether and other organic solvents to extract carotene and vitamin A from citrated human plasma (prepared from a mixture of 9 vol. of blood and 1 vol. of 3.8% trisodium citrate) under various conditions—adjustment of pH over a wide range, freezing in presence of the solvent, addition of protein precipitants, addition of aqueous colloidal solution of carotene, etc. Under all suitable conditions, comparison was to be made with simple aqueous colloidal solutions of carotene prepared as described by Van den Bergh & Müller (1920). These experiments are described later in the paper. The methods of extracting and determining the carotene and vitamin A were common to all.

Extraction

The standard method was to make three successive extractions of the material (plasma, serum, precipitated protein, etc.) with peroxide-free ethyl ether or other solvent. For each extraction, 2 vol. of solvent were added to 1 vol. of the material, and the mixture was shaken vigorously by hand for 10 min., after which the ether layer was allowed to separate spontaneously or by centrifuging and was removed. From the combined extracts, the ether was completely distilled in an atmosphere of CO₂ and with avoidance

of over-heating. The residue was taken up quantitatively in 50 ml. light petroleum, and this solution was washed once with 25 ml. of 3% KOH and then twice with 50 ml. distilled water. The light petroleum solution was then dried over anhydrous Na_2SO_4 and a sample was evaporated to a small volume in an atmosphere of CO_2 and made up to exactly 10 ml. This contained a known fraction of the carotene and vitamin A extractable by the solvent originally used, and in it these substances were determined.

To determine the carotene and vitamin A remaining unextracted by the organic solvent used, the residual material after the three extractions was mixed with an equal volume of aldehyde-free ethanol (Dunlop, 1936) and again extracted three times with 2 vol. of light petroleum, the conditions of extraction being as before. The combined extracts were concentrated to 50 ml. in an atmosphere of CO_2 and were then washed and further concentrated as described.

In each series of extractions the third contained negligible amounts of carotene and vitamin A so that extraction was complete under the conditions. It is not certain that the addition of ethanol allowed the extraction of all the vitamin A and carotene present in plasma, though it has been tentatively assumed that it did. Certainly this seemed to be true of aqueous colloidal solutions of known approximate concentration. The point, though of importance in relation to the absolute determination of carotene and vitamin A in plasma, is relatively unimportant in the experiments described in this paper.

Ethyl ether was the solvent generally used for the initial extraction of plasma or serum since other solvents caused emulsification to an extent which made the subsequent separation difficult or even impossible. The method used, however, ensured that all the carotene estimations were done in the same solvent, light petroleum.

used and the calibration curve was prepared from the 2-naphthoate of vitamin A supplied by British Drug Houses, Ltd. for purposes of standardization.

Cholesterol was determined by the Liebermann-Burchard reaction, again using the absorptiometer (no. 1 filters*) calibrated on chemically pure cholesterol. It may be noted that chloroform solutions of carotene, though not of vitamin A, give a blue-green colour with acetic anhydride in presence of a trace of sulphuric acid; this colour, however, is maximal within a few seconds and then fades so rapidly that it does not interfere with the determination of cholesterol.

Lipoid phosphorus was determined by the method described by Stewart & Hendry (1935) except that the final colour was measured on the absorptiometer (red filter* no. 1) calibrated against pure disodium hydrogen phosphate.

Plasma proteins were determined by the micro-Kjeldahl method, trichloroacetic acid being used for complete protein precipitation, and saturation with magnesium sulphate for precipitation of globulins.

RESULTS

The effect of pH on the extractability of carotene and vitamin A from plasma and from aqueous colloidal solution

Alkali or acid, in arbitrarily chosen amounts, was carefully added to the plasma, and the pH of each mixture was measured (Beckmann pH meter) without any further attempt to adjust it accurately to a predetermined figure. Since, however, small differences of pH were without pronounced effect, this method was sufficiently accurate for our purpose. Extraction of the carotene and vitamin A

Table 1. *Effect of pH on the extraction of carotene and vitamin A by ethyl ether from citrated human plasma*

(The figures represent percentages of the total amounts extractable in the presence of ethanol.)

Carotene extracted (% of total present) at pH							Vitamin A extracted (% of total present) at pH						
10-9	9-8	8-7	7-6	6-5	5-4	4-3	10-9	9-8	8-7	7-6	6-5	5-4	4-3
9	—	16	—	—	—	91	72	—	68	—	—	—	75
—	—	28	—	79	—	80	—	—	68	—	90	—	91
—	24	9	100	100	89	—	—	33	22	87	100	93	—
—	25	20	39	58	—	—	—	59	59	70	83	—	—
40	—	40	60	—	97	—	65	—	48	83	—	96	—
—	—	37	—	—	100	—	—	—	55	—	—	100	—
—	24	26	31	68	—	—	—	74	57	80	99	—	—
—	—	19	37	45	—	—	—	—	95	98	83	—	—
—	—	20	31	69	—	—	—	—	60	76	86	—	—
Mean	25	24	24	49	70	98	68	55	59	82	90	98	83

Determination

Carotene was determined by the yellow colour measured on a Spekker photoelectric absorptiometer, with blue filters* (no. 6) and a calibration curve prepared from β -carotene (British Drug Houses, Ltd.).

Vitamin A was determined by the Carr-Price reaction using Yudkin's (1941) technique. Red filters* (no. 1) were

* The filters were of the ordinary set supplied with the instrument (4455).

was done as previously described. Table 1 summarizes the results of nine such experiments.

It is evident that, at the original pH of the plasma, which was usually about 7.6, the extractability by ether of both carotene and vitamin A varies considerably, but that it is always incomplete, and is invariably greater for the vitamin than for its precursor. Increased alkalinity has very little effect on the power of ether to extract either substance, but, when the pH of the plasma is lowered,

the percentage of both vitamin A and carotene extractable by ether increases considerably, becoming maximal about pH 5, i.e. at or about the isoelectric point of the plasma proteins. When the pH falls still further, the substances become somewhat less completely extractable by ether.

Aqueous colloidal solutions of carotene behaved somewhat similarly. Table 2 gives the results of a typical experiment. The degree of dispersion of carotene in such solutions varies considerably (depending upon, *inter alia*, the proportions of carotene, acetone and water used in the preparation), and from some such solutions the whole of the carotene is readily extracted by ether at pH 7-8, a phenomenon which we have never found to occur with plasma in several hundred such extractions.

Table 2. *Effect of pH on the extraction of carotene by ethyl ether from an aqueous colloidal solution*

pH	Percentage of total carotene extracted
7.13	24
6.50	35
6.04	39
5.62	52
2.50	100

In a small number of experiments, a direct comparison was made between the behaviour of colloidal carotene solutions in water and similar solutions containing 1 % serum albumin. These are not strictly comparable with those already described, since only a single extraction with ether was made, and shaking was continued for only 5 min. Table 3 shows for a typical experiment of this kind that, at every pH tested, the presence of the protein reduced the amount of carotene extracted.

Table 3. *Effect of pH on the extraction of carotene by ethyl ether from an aqueous colloidal solution and from a similar solution containing 1 % serum albumin*

pH	Carotene extracted (% of total present)	
	From water	From 1 % serum albumin
8.7	48	11
7.6	—	14
7.0	52	—
6.3	91	—
3.0	100	16

Although plasma and aqueous colloidal solutions of carotene behave so similarly, these experiments fail to refute the idea of a protein-carotene and a protein-vitamin A complex. Indeed the comparison of the colloidal carotene solution with and without serum albumin lends some support to the idea. It is significant too that in plasma maximal extractability of both carotene and vitamin A occurs near

the isoelectric point of the plasma proteins, and that it falls off on the acid side of this point. It is therefore worth considering some observations on the effect of the experimental procedure on the proteins themselves.

Both vigorous shaking and the presence of ether are capable of producing denaturation of protein, and it was readily seen in all the experiments with plasma (which involved these two factors) that the aqueous phase developed an opalescence which was more marked in the plasma with lowered pH. To test whether this was due to protein precipitation, and to put the matter on a quantitative basis, citrated plasma of known protein content was put through the experimental procedure of pH alteration and shaking with ether, then submitted to diminished pressure for 3 hr. to remove the ether, and finally filtered, protein being determined in the filtrate. Table 4 shows that appreciable quantities

Table 4. *Effect of pH adjustment and shaking with ether on the protein content of citrated human plasma*

pH	Protein remaining in solution		Protein precipitated (%)
	(g./100 ml.)	(% of original)	
10.0	5.08	83.7	16.3
8.0	5.93	97.9	2.1
6.8	5.29	87.3	12.7
5.5	4.98	82.0	18.0
4.7	4.76	78.5	21.5

of protein had been denatured and precipitated and that these quantities were greater the nearer the pH of the plasma approached the isoelectric point of the plasma proteins. Further, fractionation with ammonium sulphate suggested that the precipitated protein was mainly, if not entirely, albumin, though the experiments were not quite clear-cut. Experiments in which the pH of the plasma was altered, and shaking with ether was omitted, indicated that it was this latter pair of factors which was chiefly responsible for the denaturation. Besides the protein actually precipitated, however, much of that remaining in the alkaline solutions had undergone denaturation. This was shown by experiments of the type summarized in Table 5. In these, after the shaking with ether and removal of dissolved ether, part of the plasma was filtered at once, and part was filtered after the pH had been adjusted to 4.7. Whereas acidification caused greater precipitation of protein, alkalization caused the greater denaturation without immediate precipitation.

These experiments demonstrated a relation between the effects of pH alteration and ether extraction on the extractability of carotene and vitamin A and on the denaturation and precipitation of plasma protein.

Table 5. *Effect of pH adjustment and shaking with ether on the protein content of citrated human plasma (a) at the pH of the shaking and (b) after readjustment of pH to 4.7*

pH	(a)		Protein precipitated (%)	(b)		Protein precipitated (%)
	Protein remaining in solution at the indicated pH			Protein remaining in solution after readjustment of pH to 4.7		
	(g./100 ml.)	(% of original)		(g./100 ml.)	(% of original)	
12.0	5.0	94.0	6.0	0.58	10.8	89.2
10.1	5.0	94.0	6.0	4.63	87.2	12.8
7.9	5.24	98.6	1.4	5.04	95.0	5.0
6.8	4.84	91.0	9.0	4.84	91.0	9.0
5.4	4.63	87.2	12.8	4.63	87.2	12.8
4.7	4.41	83.0	17.0	4.51	84.9	15.1

It is interesting to note that the extractability of cholesterol and phospholipins by ether from plasma is influenced by pH in a way very similar to that found for carotene and vitamin A. Table 6 gives the result of a typical experiment in which all four substances were determined in ether extracts made in the usual way; the amounts extracted are expressed as percentages of the total amounts found in the original plasma.

Table 6. *Effect of pH on the extraction of cholesterol and phospholipins from citrated human plasma*

pH	Amount extracted from 100 ml. plasma			
	Carotene (μg.)	Vitamin A (i.u.)	Cholesterol (mg.)	Lipoid P (mg.)
7.65	14	60	43	0.40
6.80	20	84	53	0.68
5.20	44	98	116	0.90

Effects of ethanol

Addition of ethanol to plasma, to aqueous colloidal carotene solutions, or to solutions of carotene containing 1% serum albumin, increases the quantity of carotene extractable by ether. In all cases, addition of an equal volume of ethanol is necessary to ensure maximal extraction of carotene. With smaller additions of ethanol, however, the effect is much greater on the simple aqueous solution than on those containing protein. Thus in one experiment the addition of $\frac{1}{2}$ vol. of ethanol to an aqueous colloidal carotene solution allowed the extraction (by ether) of 65% of the carotene present, whereas direct ether extraction removed only 9%. In a parallel experiment with carotene in 1% albumin, a similar concentration of ethanol increased the amount of ether-extractable carotene only from 8 to 14%. The results with plasma are similar to these latter.

These results, showing a qualitative similarity, but a quantitative difference between simple aqueous colloidal solutions of carotene and those containing protein, again show that protein has an important effect on the ether-extractability of carotene, such

as is most readily explained by supposing a carotene-protein compound of some kind.

The effect of bile salts

Drummond & McWalter (1935) mention their preparation of a carotene-deoxycholic acid complex, and speak of the absorption of carotene from the intestine as due largely to the formation of such water-soluble complexes.

Such complexes, if they were formed in plasma to which bile salts were added (whether from pre-existing free carotene or from carotene liberated during the manipulation) might be expected to cause a reduction in the amount of carotene extractable by ether. This is actually the case (Table 7). It is evident that the influence of bile salts, though not great, is appreciable, and is exerted on vitamin A as well as on carotene. When they are added to an aqueous colloidal solution of carotene, the inhibition of ether extraction is very much greater.

Table 7. *Effect of bile salts on the extraction of carotene from citrated human plasma and from aqueous colloidal solution, and the extraction of vitamin A from plasma by ethyl ether*

Bile salts (mg./100 ml.)	Carotene extracted (% of total present) from		Vitamin A (% of total present) extracted from plasma
	Plasma	Colloidal solution	
0	32	81	82
40	29	—	70
100	16	4.5	66

The effect of the protein of the plasma is very marked. The results may be interpreted by supposing the formation of a bile salt carotene (or vitamin A complex) in the presence of free carotene but not in the presence of a carotene-protein complex. Then, given a sufficiently high concentration of bile salt, practically all the (free) carotene of the aqueous colloidal solution may be supposed to enter into combination and to become almost or quite ether-insoluble. With the plasma, however, but little carotene is free, most being in combination with

protein, so that only this small amount, together with that liberated during the shaking with ether, is capable of combining with bile salt (and even here, there may be supposed to be competition between the ether and the bile salts); hence the already small proportion extracted by ether is not greatly decreased.

Effect of freezing with ether

Macfarlane (1942) has shown that when plasma or serum is shaken with ether, and the mixture is frozen at -25° , the ether layer, after re-thawing, contains a considerable amount of lipid material—very much more than can be extracted by ether when it is simply shaken with liquid plasma. Similarly, the amount of carotene and vitamin A extractable from plasma by ether is greatly increased if the ether-plasma mixture is frozen at -25° for 2 hr. and then thawed (Table 8). It is necessary to freeze the ether and plasma together; extraction of plasma which has been frozen and thawed at room temperature yields the same amount of carotene and vitamin A as extraction of the untreated control. Time appears to be a factor also, since rapid freezing of the ether-plasma mixture in liquid air was without effect.

Table 8. *Effect of freezing the ether-plasma mixture at -25° on the extraction of carotene and vitamin A from citrated human plasma by ethyl ether*

Amount extracted			
Without freezing		With freezing	
Carotene ($\mu\text{g.}$)	Vitamin A (i.u.)	Carotene ($\mu\text{g.}$)	Vitamin A (i.u.)
3	34	13	73
2	9	35	101
0	0	16	52

It should be noted that, in these freezing experiments, the plasma and ether were merely shaken together sufficiently for mixing to occur, and that after re-thawing the ether layer was separated without further shaking. This contrasts with the previously described experiments in which shaking was vigorous and was prolonged for 15 min.

With vigorous shaking, ether at room temperature and at pH 7.7 caused precipitation of about 2% of the total plasma protein (and the denaturation without precipitation of a further 3%). When the ether was merely allowed to remain in contact with the plasma at room temperature for 2 hr., the amount of protein denatured and precipitated was too small to be measured. When, however, the ether-plasma mixture was frozen for 2 hr. and re-thawed, 5–6% of the total protein was precipitated, and this appeared to be entirely albumin. Hence, as in the case of pH change, increased solubility of

carotene and vitamin A in ether has occurred in conditions which produce protein denaturation. It must, however, be recorded that an aqueous colloidal solution of carotene behaved similarly, all the carotene being extracted by ether which remained in contact with the solution for 2 hr. at -25° , while only one-third was extracted by simple shaking without freezing.

Effect of precipitation of protein by ammonium sulphate

In these experiments protein was partially or completely precipitated from the plasma by addition of ammonium sulphate to the appropriate concentration. The precipitates were separated by centrifuging and were washed with ammonium sulphate solution of the concentration used in the precipitation. Carotene and vitamin A were determined in both supernatant fluid and precipitate in the usual way (i.e. after extraction with ether in the presence of excess ethanol).

The protein precipitated by 50% saturation with ammonium sulphate contained no carotene and vitamin A, which remained completely in the supernatant fluid, from which, however, they were only partially extractable by shaking with ether.

On the other hand, the protein precipitated by 75 or 100% saturation with ammonium sulphate contained all the carotene and vitamin A, none remaining in the supernatant fluid; simple extraction with ether of the precipitated protein dissolved or suspended in water, however, yielded very little of either substance.

It seems, therefore, that the carotene and vitamin A are associated with the plasma albumin, and that this association is maintained when the albumin is precipitated without denaturation.

Aqueous colloidal solutions of carotene behaved quite differently; from one such solution at pH 7.3 only 10% of the carotene present was extractable by simple shaking with ether, whereas 76% became extractable when the solution was saturated with ammonium sulphate.

Extractability of carotene from plasma plus aqueous colloidal solution of carotene

A sample of fresh citrated plasma was extracted with ether (three extractions, each with 2 vol. of ether and shaking for 10 min.); carotene was determined in the combined extracts and (after addition of excess ethanol) in the residual plasma.

To a second sample of the same plasma, and to a third previously extracted with ether as just described, aqueous colloidal carotene solution ($\frac{1}{2}$ vol.) was added, and these were then extracted with ether, carotene being determined as before in extracts and residual plasma.

As appears from the figures given in Table 9, a large proportion (80 %) of the carotene added to the fresh plasma is not extractable by ether, but if the plasma has previously been shaken thoroughly with ether, a much smaller proportion (20 %) of the added carotene resists ether extraction. When, using the same plasma, the experiment is repeated with the pH lowered to below 7.0, practically none of the added carotene is incapable of ether extraction.

free carotene and in the other with a carotene-containing (and a vitamin A-containing) compound. We believe that this is so, and that protein is the second component of the compounds.

In support of this view, apart from the suggestive quantitative differences just mentioned, is the fact that the addition of serum albumin to aqueous carotene solutions gives a mixture which closely simulates plasma. Further, there are the very im-

Table 9. *Extraction of carotene with ether from plasma containing added carotene*

	Carotene ($\mu\text{g.}/100\text{ ml.}$)			Expected total
	Extracted	Residual	Total	
Plasma alone, pH 7.6	40	52	92	—
Plasma + 500 $\mu\text{g.}$ carotene/100 ml., pH 7.6	136	460	596	592
Extracted plasma + 500 $\mu\text{g.}$ carotene/100 ml., pH 7.6	412	152	564	552
Plasma alone, pH 6.7	72	20	92	—
Extracted plasma + 500 $\mu\text{g.}$ carotene/100 ml., readjusted to pH 7.6	520	12	532	520
Plasma alone, pH 5.6	88	4	92	—
Extracted plasma + 500 $\mu\text{g.}$ carotene/100 ml., readjusted to pH 7.6	476	6	482	504

These results suggest that carotene forms a fairly stable complex with plasma protein and that this complex is disrupted by procedures which, like shaking with ether, denature the protein.

DISCUSSION

Certain facts concerning the extraction of carotene (and vitamin A) from citrated human plasma are clear: the amount extracted is a function of pH, increasing as the pH is decreased from the normal and becoming maximal at pH 4–5 but not altering very much with increased alkalinity; a similar increase is produced by the addition of ethanol, maximal when the volume added is about equal to the volume of plasma; freezing the plasma at -25° in contact with ether also produces an increase in the amount of carotene extracted; saturation of the plasma with ammonium sulphate precipitates the carotene and vitamin A with the proteins but without rendering it ether-soluble, while half-saturation neither precipitates these substances with the globulins nor renders them ether-soluble; bile salt in sufficient concentration decreases the amount of carotene and vitamin A extractable by ether to a small but measurable extent.

Qualitatively, similar results are obtained with aqueous colloidal solutions of carotene except by addition of ammonium sulphate, which increases the ether-solubility of the carotene. As has been shown, however, the results with such aqueous solutions are quantitatively different from those with plasma (or with aqueous solutions to which serum albumin has been added), and the question is whether these differences are sufficient to justify the view that we are dealing in the one case with

portant facts that ammonium sulphate in suitable concentrations precipitates carotene and vitamin A with the plasma albumin and that ether does not extract these substances from the redissolved or suspended precipitate any more readily than from the original plasma. Add to all this the observations that the combined effect of shaking with ether and altering the pH of plasma denatures and precipitates protein as it alters the extractability of carotene and vitamin A, and that extraction in the presence of ethanol only becomes complete when the ethanol concentration is sufficient to cause protein precipitation. Finally, there is the evidence that carotene in aqueous colloidal solution added to plasma becomes less ether-extractable than would be expected, whereas it is readily extracted if added to plasma of which the proteins have previously been partly denatured by shaking with ether near their isoelectric point. Against all this there can only be set the qualitative similarity of behaviour between plasma and aqueous colloidal carotene solutions.

If this conclusion of the existence of a carotene-protein and a vitamin A-protein compound be accepted, then the evidence indicates that the protein concerned is plasma albumin. Bennhold (1938) states that in cataphoretic migration carotene moves with the serum globulin, and adversely criticizes fractional precipitation as a means of studying plasma proteins. In view of this it is safer to regard the identity of the protein combined with carotene and vitamin A as 'non proven', but there is a little further evidence suggestive of its being albumin. Padersen & Waldenstrom (1937) have shown by studies employing electrophoresis and the ultracentrifuge that bilirubin in plasma is bound to albumin, and they have further shown that bilirubin

added to solutions of serum albumin is bound, while with ovalbumin it remains free. Now when carotene and vitamin A are extracted from plasma, the extracts are contaminated with bilirubin (which is removed by washing with 3% KOH solution). When the pH of the plasma is altered, the amounts of carotene and bilirubin increase proportionately, and become maximal at the same pH. Moreover, there is the experimental finding that conditions which favour the ether extraction of carotene and vitamin A are those which produce denaturation and precipitation of protein, the protein concerned being mainly if not entirely albumin.

SUMMARY

1. Only small amounts of carotene and vitamin A are extracted from citrated plasma by shaking with ether; lowering the pH allows the extraction of greater amounts, the maximum (probably all) being extracted at pH 4-5. The shaking with ether causes denaturation and precipitation of protein, this effect also increasing as the pH is decreased.

2. The presence of ethanol increases the extractability of carotene and plasma; in a mixture of equal volumes of plasma and ethanol, extraction appears to be complete.

3. Bile salts decrease the amounts of carotene and vitamin A extracted by ether.

4. Half saturation of plasma with ammonium sulphate causes no precipitation of carotene and vitamin A nor change in their ether-solubility; three-quarter or complete saturation precipitates these substances with the albumin, but they are not readily extracted from the precipitate by ether.

5. Freezing plasma at -25° in contact with ether causes an increase in the amount of carotene and vitamin A extracted by the ether.

6. Carotene in aqueous colloidal solution behaves qualitatively like plasma in these various ways, but there are important quantitative differences.

7. Carotene in aqueous colloidal solution added to normal plasma becomes unextractable by ether, but this does not occur when plasma previously shaken with ether (so that protein has been denatured) is used.

8. The evidence is held to indicate the existence of compounds of carotene and vitamin A with protein, the protein concerned being probably, but not certainly, albumin. Denaturation and precipitation of the protein appears to weaken or disrupt the compounds.

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NOTE BY C. P. STEWART. At the time of Dr Mystkowski's death a year ago, our experiments had, in our joint opinion, led to the conclusion that carotene and vitamin A exist in plasma as protein compounds of some kind. Later experiments, in the planning of which Dr Mystkowski shared, have further supported this conclusion and led to the

suggestions that albumin is the protein concerned, and that denaturation affects the stability of the compounds. I believe that we have not made any claims in this paper with which Dr Mystkowski would not have agreed, but the responsibility for any errors must, of course, rest with the surviving authors.